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EXPLORATION OF ANTIMUTAGENIC PROPERTIES OF METHANOLIC EXTRACT OF LEAVES OF *DIPTERACANTHUS PATULUS* (JACQ.) NEES

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ABSTRACT

Mutations are the cause of innate metabolic defects in cellular systems, triggering morbidity and mortality in living organisms. The mutagens are involved in the initiation and promotion of several human diseases, including cancer. Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products. The present study focuses on the investigation of antimutagenic effect of leaves of *Dipteracanthus paulus* (Jacq.) Nees by Ames Assay. The genotypes of the *Salmonella typhimurium* strains TA 98 and TA 1535 was confirmed by Histidine requirement, *rfa* Mutation, *uvrB* Mutation and Presence for R-Factor. The Toxicity Test indicated that the leaves of *Dipteracanthus patulus* were non toxic to *Salmonella typhimurium* strains. The Methanolic extract of Leaves of *Dipteracanthus patulus* was tested for their antimutagenic activity against the sodium azide (NaN3) induced mutagenicity in the tester strains TA 98 and TA 1535. Methanolic extract of Leaves of *Dipteracanthus patulus* showed decrease in the number of revertants colonies against sodium azide induced mutagenicity by TA 98 and TA1535. The results of the present study indicated that Methanolic extract of leaves of *Dipteracanthus patulus* showed strong antimutagenic activity against the standard mutagen action.

KEYWORDS: *Dipteracanthus patulus*, *Salmonella typhimurium* Strains TA 98 and TA 1535, Antimutagenic Study

INTRODUCTION

Mutations are the cause of innate metabolic defects in cellular systems, triggering morbidity and mortality in living organisms. The mutagens are involved in the initiation and promotion of several human diseases, including cancer[1]. Mechanism of mutagenesis is complex however many mutagens and carcinogens may act through the generation of reactive oxygen species. ROS may play a major role as endogenous initiators of degenerative processes, such as DNA damage and mutation, which may be related to cancer, heart disease and aging[2].

Somatic gene mutations are the basic events for the conversion of a normal cell to a mutant cell. This mutant cell converted to malignant cell through several genetic changes. Several chemicals have been implicated in cancer causation. Some of these are sodium azide, ethidium bromide, hydroxyl amine, Methylnitronitrosoguanidine (MNNG), N, N' bis-(1-naphthyl) N, N'-diphenyl -1, 1 biphenyl-4, 4'-diamine (α-NPD), etc.[3].

There is a continuous search for new medical preparations against a great number of ailments, including cancer. This is because existing medication often has unwanted side effects or because of loss of efficiency in the long run.

Epidemiological studies indicate that many cancers are dependent on multiple mutational etiology, as well as on inherited mutator phenotype. The search for inhibitors of mutagenesis may therefore be useful as a tool to discover anticarcinogenic agents[4].

Chemotherapy and radiotherapy, the conventional cancer treatments used nowadays, are expensive and cause many side effects, including such minor ones as vomiting, alopecia, diarrhea, constipation, and major ones such as myelosuppression, neurological, cardiac, pulmonary and renal toxicity. All such side effects reduce the quality of life and discourage patients to observe medication protocols which then lead to the progression of cancer and associated complications. In addition, many of these treatments present limited anti-cancer activity[5]. Resection surgery procedures, on the other hand, cause functional deficiencies or esthetic discomfort. Therefore, there is a need to discover alternative anticancer drugs, hopefully more potent, as well as more selective and less toxic than those currently in use. Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products[6].

Dipteracanthus patulus (Jacq.) Nees. (Syn. *Ruellia patula* Jacq). (Acanthaceae) is a medicinal herb traditionally used in the treatment of wounds in the rural areas. The leaves are used for treating itches, insect bites, paranychia, venereal diseases, sores, tumours, rheumatic complaints and eye diseases. It is cardiotonic and single drug remedy for against the deadly poison of kaduva chilanthi (Tiger Spider) by kani tribes in agasthiarmalai[7,8]. The methanolic extract of *Dipteracanthus patulus* (Jacq.) Nees has shown promising antimicrobial and hepatoprotective activity. Leaves of this plant are used to cure liver complaints by the peoples of Sholapur region (MS), India[9]. Hence the present study focuses on the investigation of antimutagenic effect of leaves of *Dipteracanthus paulus* (Jacq.) Nees.

MATERIALS AND METHODS

Salmonella Mutagenicity Test (Ames Assay)[10]

Culture Preparation

Salmonella typhymurium strains TA 98 and TA 1535 were used for this experiment. TA 1535 detected with mutation by base pair substitution; TA 98 detected with frame shift mutations. Frozen cultures of the tester strain were stored at- 20°C. A fresh nutrient broth culture was grown to a density of 1-2 X 10^9 cells/ml and for each 1 ml of culture, 0.09 ml of di-methyl sulphoxide was added as cryoprotective agent. The bacterial culture was inoculated in fresh nutrient broth and grown for 12 hour at 37°C before each experiment.

Confirming Genotypes of the Salmonella Strains

Histidine Requirement

The histidine character of the tester strains TA 98 and TA 1535 were confirmed by demonstrating the histidine requirement for growth on selective agar plates such as histidine/biotin plate. Biotin was also required by all of the tester strains because of the *uvrB* deletion which extended through the bio-gene. Cotton swab was dipped in the 12 hour broth culture and sweep was made across the histidine/biotin plate. Then, the plates were incubated overnight at 37°C and the growth was examined on the next day.

rfa Mutation

Strains having the deep rough (*rfa*) character were tested for crystal violet sensitivity. 0.1 ml of fresh overnight culture of the tester strains (TA 98 and TA 1535) was added to a test tube containing 2 ml of moltent agar at 45°C. The top

agar tubes were vortexed for 3 sec at low speed and poured on nutrient agar plate without histidine and biotin. The plates were tilted and rotated for the even distribution of the top agar on the plates. The plates were placed on a leveled surface and allowed several times for agar to become firm. 10µl of 1 mg /ml solution of crystal violet was pipetted to the centre of the sterile disc (1/4 inch) and discs were transferred to each of the inoculated plates using sterile forceps. The discs were lightly pressed with forceps to embed it slightly in the overlay. The plates were incubated at 37°C and observed for crystal violet sensitivity.

uvrB Mutation

The *uvrB* mutation was confirmed by demonstrating UV sensitivity in strain that contain this mutation. The R-factor strain TA 98 and non R-factor TA 1535 were streaked with sterile swabs across the nutrient agar plate. A half of one was covered with aluminum foil, and it was exposed to UV radiation 8 seconds. The irradiated plates were incubated at 37°C for 12-24 hours.

R-Factor

The R-factor strains TA98 were tested for the presence of the ampicillin resistance factor. To test for ampicillin resistance, the cultures were streaked across the half of an ampicillin plate using the procedure as described for confirming the histidine requirement. The non R-factor strain TA 1535 was tested on the same plate as a control for ampicillin activity.

Toxicity Test

For the testing the toxicity minimal glucose agar medium was prepared. The plant extract was added to the minimal glucose agar and mixed well. Minimal glucose agar containing plant extract was poured onto the petridish. Overnight culture of strains TA 98 and TA 1535 was streaked onto it. The plates were incubated for 24 hours.

Determination of Antimutagenicity of Methanolic Extract of Leaves of *Dipteracanthus patulus* against Direct Acting Mutagen

Antimutagenicity of Methanolic extract of leaves of *Dipteracanthus patulus* was tested in *Salmonella typhymurium* strains TA 98 and TA 1535 using direct acting mutagen.

Plate incorporation method was done for antimutagenicity assay without microsomal activation. Fresh cultures of S*almonella typhymurium* strains TA 98 and TA 1535 (1-2x10^9 cells/ml) were mixed with 2 ml of molten agar containing 0.5mM histidine/biotin solution, different concentration of plant extract (0.5-2 mg/plate) and direct acting mutagen sodium azide (2.5µg/plate). Further it was spread over minimal glucose agar plates. Plates were incubated for 48 hours at 37°C and the relevant colonies were counted.

% inhibition = [1-T /M] ×100

T is No. of revertants per plate in presence of mutagen Methanolic extract of Leaves of

Dipteracanthus patulus

M is No. of revertants per plate in positive control

The antimutagenic effects of the extract were graded according to percentage inhibitory effect by the extract. When percentage inhibition of mutagen by plant extract was more than 40%, 25-40 and less than 25%, the antimutagenic effect considered strong, moderate and weak respectively[11].

RESULTS AND DISCUSSIONS

The *Salmonella typhimurium*/microsome assay (*Salmonella* test; Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. The test uses a number of *Salmonella* strains with preexisting mutations that leave the bacteria unable to synthesize the required amino acid, histidine, and therefore unable to grow and form colonies in its absence. New mutations at the site of these preexisting mutations, or nearby in the genes, can restore the gene's function and allow the cells to synthesize histidine. These newly mutated cells can grow in the absence of histidine and form colonies.

For this reason, the test is often referred to as a "reversion assay." The *Salmonella* strains used in the test have different mutations in various genes in the histidine operon; each of these mutations is designed to be responsive to mutagens that act *via* different mechanisms. Additional mutations were engineered into these strains to make them more sensitive to a wide variety of substances. The mutation values obtained can only be expressed as number of mutants/plate, or number of mutants/amount of chemical added [12].

It is recommended that the tester strains be analyzed for their genetic integrity and spontaneous mutation rate when frozen cultures are prepared. A strain check should also be performed whenever an experiment is performed. The strain check is usually performed with the nutrient broth overnight cultures. Histidine dependence (his), *rfa* marker, *uvrB* deletion and Presence of plasmid pKM101 (ampicillin resistance) should be followed for a complete strain check[13].

Confirming Genotypes of the *Salmonella typhimurium* Strains TA 98 and TA 1535

Histidine Requirement

The growth of *Salmonella typhimurium* TA98 and TA 1535 strains was observed on histidine-biotin plates after 24 hours incubation at 37°C. The presence of colony in biotin-histidine medium and absence in control biotin medium showed that these strains were dependent to histidine.

rfa Mutation

The existence of inhibitory zone around of the disk indicated that the tester strain TA 98 and TA 1535 did not grow and the *rfa* mutation was occurred. This mutation caused relative decrease of lipopolysaccharide barriers and then, increased the cell wall permeability for bigger molecules like crystal violet.

uvrB Mutation

The TA 98 and TA 1535 strains which showed lack of growth in radiated culture region indicated that *uvrB* mutation was occurred.

R-Factor

Growth was observed in the ampicillin plates with TA 98. No growth was observed for TA 1535. TA 98 is an R-factor strain. This strain possessed pKM101, Plasmid DNA essential for ampicillin resistance. No growth was seen on

TA 1535 as it had no R-factor and it was tested as a control for ampicillin sensitivity. The R-factor served as a convenient marker that made it possible to test for the presence of plasmid.

Toxicity Test of Methanolic Extract of Leaves of *Dipteracanthus patulus* on *Salmonella typhimurium* Strains TA 98 and TA 1535

The Growth of *Salmonella typhimurium* strains TA 98 and TA 1535. Methanolic leaves extract of *Dipteracanthus patulus* were non toxic to *Salmonella typhimurium* strains at the tested concentration 2mg/plate.

Antimutagenic Activity of Methanolic Extract of Leaves of *Dipteracanthus patulus* against Sodium Azide Induced Mutagenicity on *Salmonella typhymurium* Strain TA 98 and TA 1535

The effect of Methanolic extract of Leaves of *Dipteracanthus patulus* on the direct acting mutagen Sodium azide (NaN3) induced mutagenicity on *Salmonella typhimurium* strain TA 98 was presented in Table 1. The effects of Methanolic extract of Leaves of *Dipteracanthus patulus* on the direct acting mutagen Sodium azide (NaN3) induced mutagenicity on *Salmonella typhimurium* strain TA 1535 was presented in Table 2.

Antimutagenic Activity of Methanolic Extract of Leaves of *Dipteracanthus patulus* against Sodium Azide Induced Mutagenicity on *Salmonella typhymurium* Strain TA 98

Table 1

Group	Average No. of Colonies Present	% Inhibition
Control	251 ± 10	-
MELDP (0.5mg/plate)	64 ± 9***	75
MELDP (1mg/plate)	23± 5***	91
MELDP (2mg/plate)	8±3***	97

MELDP- Methanolic extract of Leaves of *Dipteracanthus patulus*

Values are expressed as mean ± SD, n = 3

Statistical analysis by one way ANOVA by Dunnett's Multiple Comparision

P Values ***P<0.001 **P<0.01, *P<0.05 compared with control

Antimutagenic Activity of Methanolic Extract of Leaves of *Dipteracanthus patulus* against Sodium Azide Induced Mutagenicity on *Salmonella typhymurium* Strain TA 1535

Table 2

Group	Average No. of Colonies Present	% Inhibition
Control	181 ± 7	-
MELDP (0.5mg/plate)	42 ± 6***	77
MELDP (1mg/plate)	11 ± 3***	94
MELDP (2mg/plate)	3 ± 1***	98

MELDP- Methanolic extract of Leaves of *Dipteracanthus patulus*

Values are expressed as mean ± SD, n =3

Statistical analysis by one way ANOVA by Dunnett's Multiple Comparision

P Values ***P<0.001 **P<0.01, *P<0.05 compared with control

The Methanolic extract of Leaves of *Dipteracanthus patulus* was tested for their antimutagenic activity against the sodium azide (NaN3) induced mutagenicity in the tester strains TA 98 and TA 1535. Methanolic extract of Leaves of *Dipteracanthus patulus* showed decrease in the number of revertants colonies against sodium azide induced mutagenicity by TA 98 and TA1535. At 0.5 mg/plate, 1mg/plate and 2mg/plate extract concentration, percent inhibition of mutagenicity for TA 98 were 75%, 91% and 97%. At 0.5 mg/plate, 1mg/plate and 2mg/plate extract concentration, percent inhibition of mutagenicity for TA 1535 were 77%, 94% and 98%. Antimutagenicity of Methanolic extract of Leaves of *Dipteracanthus patulus* was found to be concentration dependent.

Mutation is an important factor in carcinogenesis. Therefore, the incidence of cancer may be reduced by decreasing the rate of mutation. The best way for humans to decrease the rate of mutation is to avoid exposure to or ingestion of mutagens and carcinogens. Cancer is considered as one of the main causes of mortality throughout the industrial world in the present century. Scientists believe that damage to the genetic material, changes in DNA sequence and continuity, mutation in genes and other genetic changes in chromosomal structures play important roles in carcinogenesis. The use of anti-mutagens and anti-carcinogens in everyday life is the most effective procedure for preventing human cancer and genetic disease. Ames mentioned that some natural substance contain factors, which act to lower the mutation rate either by inactivating mutagens or interfering in the process of mutagenesis. The antimutagenic substances may prevent cancer because they can destroy mutagens both inside and outside body cells, and block mutagens that damage DNA and cause mutations in cells. Today, bacteria are being used for the assessment of antimutagenic activities of different compounds in a short-time with excellent results. One of the methods used for assessing the mutation prevention properties of a compound in bacteria is the Ames test. Ames test is a worldwide short-term bacterial reverse mutation test specifically designed for screening a variety of new chemical substances and drugs that can produce genetic damage that leads to gene mutations. The Salmonella strains used in the test have different mutations in various genes in the histidine operon, each of these mutations is designed to be responsive to mutagens that act via different mechanisms[14].

CONCLUSIONS

The results of the present study indicated that Methanolic extract of leaves of *Dipteracanthus patulus* showed strong antimutagenic activity against the standard mutagen action. The possible mechanism may be the inhibition of interaction between genes and biochemically reactive mutagens, interaction with the pro-mutagens making them unavailable for the enzymatic process and free radical scavenging activity. Since the extract showed antimutagenic activity it can possess chemopreventive mechanisms.

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